Unmediated amperometric enzyme electrodes

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GEORGE S. WILSON and DANIEL R. THÉVENOT

1. Introduction

Since the development of the enzyme-based sensor for glucose first described by Clark (1) in 1962, there has been an impressive proliferation of applications involving a wide variety of substrates. These applications have recently been extensively reviewed elsewhere (2–4). These applications involve enzymes which catalyse redox reactions whose rates are made proportional to the analyte (substrate) concentration. Typically the progress of the reaction is monitored by measuring the rate of formation of a product or the disappearance of a reactant. If the product or reactant is electroactive, then its concentration may be monitored directly. The enzymes catalysing these reactions are typically oxidoreductases, but hydrolytic enzymes such as alkaline phosphatase can also be used if they produce an electroactive species. Because the species usually involved are small molecules, they can be monitored amperometrically without the need for a mediator, hence sensors based on these reactions are called 'unmediated amperometric enzyme electrodes'. The most common system by far involves the monitoring of the disappearance of oxygen or the appearance of hydrogen peroxide (2). Strictly speaking, a biosensor should be 'reagentless' meaning that no additional reagents need be added to make the sensor function. Oxygen is, of course, a reagent which is consumed in the reaction, but because it is usually already present in the sample, no reagent addition is required. A large number of enzymes use NAD^+ or NADH as a cofactor, which must be added to the solution. Regeneration of the cofactor within the sensor has not proven easy to implement and its electrochemistry is also not straightforward. See Chapter 3 for further details on NADH electrochemistry.

Although beyond the scope of this presentation, it is also possible to incorporate several enzymes into the same sensor. There are three reasons for doing this.

(a) Conversion of an analyte by a sequence of reactions into a form that can be conveniently detected electrochemically.
(b) Conversion of interferences in the sample into electrochemically or enzymatically inactive forms.
(c) Recycling of reactants to enhance enzymatic turnover.

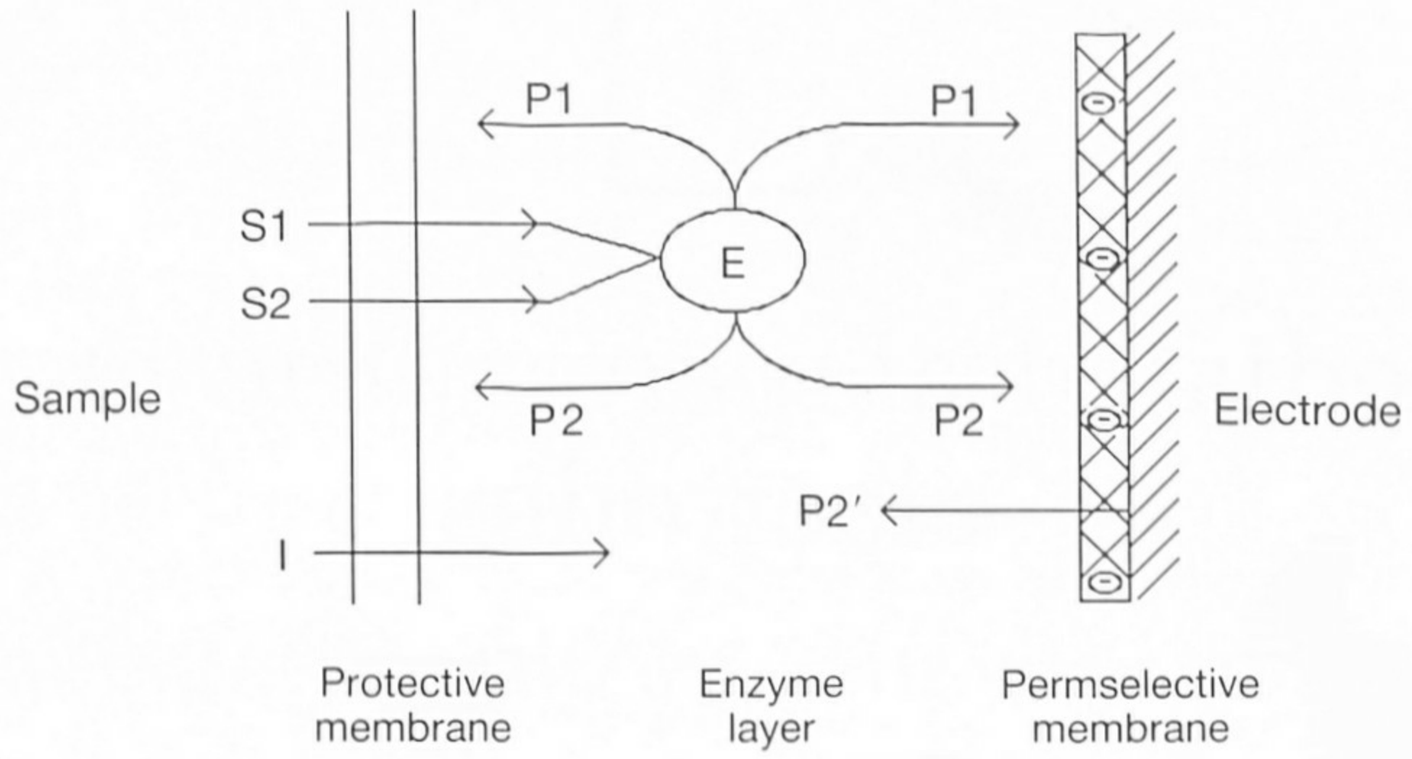

Figure 1. Schematic profile of amperometric enzyme electrode. S1,S2, substrates; P1,P2, products; P2′, product of electrochemical reaction of P2; I, interference (neutral or charged); E, enzyme.

The reader is referred to a recent review by Scheller (5) on this subject.

A schematic diagram of an enzyme electrode is shown in *Figure 1*. The electrode sensing element is usually constructed of platinum, but gold and various forms of carbon have also been used. Immediately adjacent to the electrode is the enzyme layer which is formed by the entrapment of the enzyme within a gel, by covalent glutaraldehyde-mediated cross-linking with a protein such a bovine serum albumin (BSA), or by covalent attachment to a support membrane. Direct attachment or adsorption of the enzyme on the electrode is also possible. The literature concerning the immobilization of biocatalysts is vast. An excellent recent monograph (6) should be consulted for details. Unfortunately, it is not possible to generalize about immobilization methods. Some methods work well with certain enzymes, but not with others. The methods which we present below are ones which are easily implemented, and which usually yield satisfactory results. The objective is to produce a layer of enzyme which is as thin as possible but with enzyme immobilized at the highest possible specific activity. Failure to do this results in sensors with poor sensitivity and long response times. The final component in the sensor is the outer (protective) membrane. This membrane serves several important functions. First, it is a protective barrier which prevents large molecules such as proteins from entering the enzyme layer. Biological fluids often contain catalase which could destroy the hydrogen peroxide produced in the enzyme layer thus leading to an erroneously low response. The membrane barrier will also prevent the leakage of enzyme into the sample solution. A properly chosen membrane exhibits permselective properties which are additionally beneficial to sensor function. At the applied potential corresponding to the oxidation of hydrogen peroxide it is also possible to oxidize a variety of amino acids as well as urate and ascorbate. However, if a membrane possessing a negative charge is employed, it can largely exclude anionic electroactive interferences (I^-) of this latter type. This is illustrated in *Figure 1* as a

permselective inner membrane. If only one membrane is employed, the outer membrane can also be permselective. Finally the membrane can serve as a diffusional barrier for the substrate itself. Most enzymes follow some form of Michaelis–Menten kinetics which leads to enzymatic reaction rates largely non-linear with concentration. Enzyme-based sensors, however, are capable of linear dynamic ranges of several orders of magnitude because the response is controlled by diffusion through the membrane and not by enzyme kinetics. If the enzyme layer activity is low, then a thick membrane will be required to achieve good linear response. This will also lead to slow response. On the contrary, if the enzyme layer activity is high, a thin outer membrane is sufficient and a rapid response may be obtained. It is important to understand the basic principles of sensor function in order to optimize response characteristics, and Chapter 9 describes some of the theoretical tools for analysing biosensor performance.

2. Basic techniques

2.1 Enzyme immobilization

The proper functioning of an enzyme-based sensor is, of course, heavily dependent on the properties of the enzyme itself. There are a number of commerical sources of enzymes including: Boehringer-Mannheim, Calbiochem, and Sigma. Commercial sources of less common enzymes may be found by consulting Linscott's Directory of Immunological and Biological Reagents. For common enzymes such as glucose oxidase (GOx EC 1.1.3.4) several grades are available. In this case not only should the specific activity be considered but also the presence of impurities such as catalase. Oxidoreductases are very sensitive to immobilization and usually yield specific activities which are 5–20% of the soluble enzyme. Three types of immobilization techniques are illustrated below which will work for glucose oxidase and probably a range of other enzymes as well.

2.1.1 Entrapment behind membrane

This example will be illustrated for the preparation of a glucose sensor and is a modification of a previously published procedure (7). The sensor probe is shown in *Figure 2a* and a method for immobilizing due enzyme is given below.

Protocol 1. Physical adsorption of enzyme

1. Prepare a mixture of 24 g of cyclohexanone, 24 g of acetone and 1 g of cellulose acetate (39.8% acetyl content, available from Aldrich Chemical Co.).
2. Stir the mixture at room temperature until the cellulose acetate has dissolved and then cast a thin film on to the surface of the sensor probe. Allow the solvent to evaporate to leave a thin film on the surface.
3. Dissolve glucose oxidase (Sigma Type II, sp. act. 25 U mg^{-1}) in 0.1 M phosphate buffer, pH 7.4 to a final concentration of 25 mg ml^{-1}. Place 20 μl of this enzyme solution on top of the cellulose acetate membrane and allow the water to evaporate (5–10 min).

Protocol 1 *continued*

4. Cover the dried enzyme layer with a 1 cm square membrane of either collagen (Centre Technique du Cuir), polycarbonate (Nucleopore) or general purpose dialysis tubing (mol wt. cut off 12 000 – 14 000. Viscase Corporation) and fit it in place with an 'O' ring. The membrane should be held as tightly as possible without tearing it.
5. Trim off the excess membrane and place the probe in a 0.1 M phosphate buffer solution, pH 7.4 for 2 h before use.

Note: A suitable polycarbonate membrane has a 0.05 μm pore size and a 10 μm thickness.

A glucose sensor prepared in this way will be usable for several months if stored at room temperature in phosphate buffer.

2.1.2 Reticulation with glutaraldehyde

In some situations a higher loading of active enzyme can be obtained if the adsorbed enzyme is cross-linked with glutaraldehyde. Thus a minor variation of *Protocol 1* can be made.

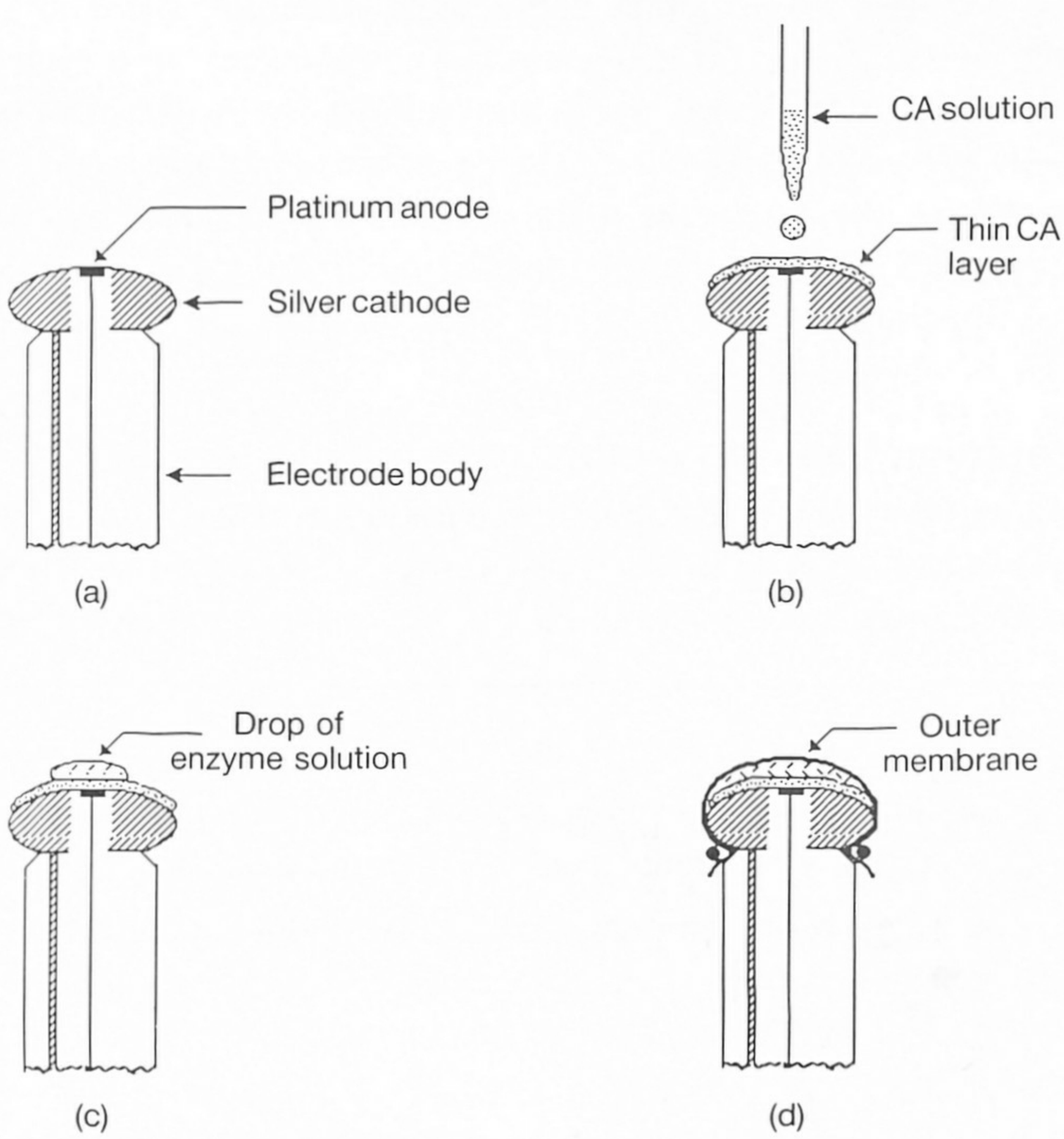

Figure 2. Preparation and immobilization of enzyme. (**a**) Sensor body; (**b**) application of CA membrane; (**c**) application of enzyme; (**d**) application of outer membrane.

Protocol 2. Reticulation of enzyme using glutaraldehyde

1. Prepare the probe exactly as described in *Protocol 1* up to step 3.
2. After the enzyme solution has dried add 10 μl of a 1% solution of glutaraldehyde. (A suitable source is Sigma Type I supplied as a 25% solution which should be stored frozen and diluted in water immediately before use.)
3. Allow the glutaraldehyde solution to evaporate and then fit the outer membrane as described in *Protocol 1*.

A variation on this reticulation procedure involves the mutual cross-linking of the enzyme with another protein such as BSA, Fraction V powder (Sigma). This procedure can lead to higher enzyme activity and greater stability.

Protocol 3. Reticulation of enzyme using glutaraldehyde and bovine serum albumin

1. Prepare the probe exactly as described in *Protocol 1* up to step 2.
2. Make the glucose oxidase solution up exactly as described in *Protocol 1* and also prepare a BSA solution (50 mg ml^{-1}) in the same phosphate buffer.
3. Mix 10 μl of each of the protein solutions and place the resulting 20 μl on the cellulose acetate membrane.
4. After 1–2 mins add 10 μl of 2.5% glutaraldehyde solution. The liquid layer should harden rapidly.
5. After a further 1–2 h fit the outer membrane as described in *Protocol 1*.

2.1.3 Covalent attachment to membrane

Covalent attachment procedures are more complicated but are especially useful in cases where the sensor is so small that the appropriate membranes must be fabricated directly on the sensing element. Under such conditions covalent procedues afford greater control over enzyme immobilization and give more stable and reproducible enzyme activity.

Collagen (acyl azide)

Collagen membranes (100 μm thick when in dry state, 300–500 μm when wet) can be obtained from the Centre Technique du Cuir, Lyon, France. Three to four membranes of 25 cm^2 total surface area can be derivatized simultaneously. This procedure has been described in detail by Thévenot and co-workers (8) and is presented below.

Protocol 4. Covalent attachment of enzyme to collagen membranes

1. Take three or four collagen membranes with a total surface area of ~25 cm^2.
2. Incubate the membranes in 50 ml of 100% methanol containing 0.2 M

Protocol 4 *continued*

hydrochloric acid for 3 days at room temperature to convert the carboxyl groups to their methyl esters.

3. Wash the membranes carefully with distilled water.
4. Incubate the membranes in 100 ml of 1% hydrazine for 12 h at room temperature and then wash with water at 0°C.
5. Prepare a solution of 50 ml of 0.5 M potassium nitrite containing 0.3 M hydrochloric acid on an ice bath.
6. Immerse the hydrazine treated membranes in the nitrous acid solution for 15 min, then wash them with a 50 mM glycine-sodium hydroxide buffer pH 9.1.
7. Place the activated membranes in 5 ml of enzyme solution (1.5 mg ml^{-1}, at least 50 Units of activity) and store at 4°C for 12 h.
8. Wash the membranes with 0.1 M phosphate buffer pH 7.4.

Note: If the collagen is exposed for too long to the acid/methanol solution the membranes become extremely fragile.

Cellulose acetate (BSA–parabenzoquinone)

In this procedure the cellulose acetate surface is oxidized to produce aldehyde groups. These are then reacted with amine functionalities on the BSA to form a Schiff base linkage which is stabilized by borohydride reduction. This creates a BSA coating on the surface to which activated enzyme is coupled through amine functions using the *p*-benzoquinone coupling procedure. This procedure increases available functionalities on the CA surface and creates a BSA coating; for method, see *Protocol 5*.

Protocol 5. Covalent immobilization of glucose oxidase to cellulose acetate membranes

1. Dissolve 1.8 mg of cellulose acetate in a mixture of 20 ml of acetone and 3 ml of water.
2. Cast 1 ml of this solution on to a clean dry glass plate using a spreader (Touzart) and allow the solvent to evaporate for 1 min at room temperature.
3. Remove the membrane by immersing the glass plate in distilled water and floating it off. The resulting membrane is cut in to smaller pieces and stored at room temperature in water.
4. Suspend four membranes (each 2.5 cm square) in 100 ml of 0.1 M sodium periodate for 20 min at room temperature.
5. Wash the membranes in distilled water for 5 min then immerse them in 10 ml of a 10 mg/ml solution of BSA in 0.1 M borate buffer, pH 9 for 2 h.
6. Remove 9 ml of the BSA solution and add 4 mg of sodium cyanoborohydride (Aldrich). Incubate at room temperature for 2 h.

Protocol 5 *continued*

7. Wash the membranes in distilled water for 5 min and then store in phosphate-buffered saline at room temperature.
8. Recrystallize *p*-benzoquinone (Merck) from petroleum ether and prepare a solution of 15 mg/ml in ethanol.
9. Add 100 μl of the freshly prepared *p*-benzoquinone to 0.5 ml of a 20 mg/ml solution of glucose oxidase in 0.1 M phosphate buffer pH 7.4 in a tube covered by aluminium foil.
10. Incubate the mixture for 30 min at 37°C and then remove the excess *p*-benzoquinone by gel filtration through a Sephadex G-25 column (1 × 10 cm) equilibrated with 0.15 M sodium chloride and operating at a flow rate of 20 ml h^{-1}. Collect the pink-brown band that elutes in the void volume (2–3 ml). For further details consult ref. 9.
11. Suspend the BSA-cellulose acetate membranes in 2–3 ml of the activated glucose oxidase solution after adjusting the pH of the latter to 8–9 with 0.25 ml of 1 M sodium carbonate. Incubate at room temperature for 38 h.
12. Remove the membranes, wash them by stirring in 0.15 M potassium chloride solution for 24 h and then store them in phosphate-buffered saline pH 7.4 containing 1.5 mM sodium azide.

Note: The spreader has four channelled surfaces which yield films of 5, 10, 15 and 30 μm thickness. A 15 μm thickness is chosen.

Activated polyamide

Coulet and co-workers (10,11) have demonstrated the utility of activated nylon membranes for enzyme immobilization. Originally designed for immunochemical applications, Biodyne immunoaffinity membranes (120 μm thick, 0.2 μm pore diameter, Pall, Glen Cove, NY 11542 USA) have been successfully used for glucose oxidase and lactate oxidase immobilization.

Protocol 6. Covalent immobilization of enzyme to Biodyne membranes

1. Cut four 8 mm disks from a 120 μm thick, 0.2 μm pore size Biodyne membrane (Pall).
2. Immerse the membranes in 1 ml of a 1.5 mg/ml solution of the enzyme in 0.1 M phosphate buffer pH 7.4 and stir for 2 h at 4°C.
3. Wash the membranes twice for 20 min each time in 1 M potassium chloride and store in 0.1 M phosphate buffer pH 7.4 at 4°C.

Note: If lactate oxidase is immobilized on polyamide membranes its storage stability can be improved by the addition of 0.1 M potassium chloride, 10 mM magnesium chloride and 10 μM FAD to the storage buffer.

2.2 Protective membranes

In general the outer protective membrane must be compatible with the medium into which it will be placed and at the same time must allow the passage of substrates and analytes. For sensors with essentially planar active surface areas greater than about 1 cm^2, pre-cast membranes can be used. These have the advantage that their properties are generally more uniform than membranes deposited directly on the sensor from solution. They are also commercially available. Their disadvantage is that if the geometry of the sensor is not planar, then it may be difficult to position the membrane so that it is in uniform contact with the sensor surface. Failure to do so can cause the response characteristics to change with time. By contrast, deposition of polymer layers from solution produces a more adherent layer which can also accommodate a miniature or spherical geometry. Generally if pre-cast membranes are less than 10−15 μm thick, they cannot be manipulated without tearing. Therefore if a thinner membrane is desired, direct deposition is again the method of choice.

In the area of pre-cast membranes, three types are commercially available and easy to use. Collagen, a hydroxylic natural protein material is processed and cast into membranes. These membranes are easy to derivatize (see *Protocol 4*) and handle. At room temperature they work well, but at physiological temperature (37°C) they soften to the point of being unstable. They are compatible with biological fluids and exclude proteins, however, no obvious permselectivity is observed. Other sources of this material are FMC, Inc., and Sigma.

Synthetic materials available from Nucleopore Corp. in pre-cast form include polycarbonate membranes 'drilled' with neutrons to produce holes of uniform and controlled size. The 0.05 μm pore size, 10 μm thick membrane is the preferred material and it is strong and easy to handle. It exhibits no permselectivity for small molecules. The Biodyne immunoaffinity membranes mentioned in *Protocol 6* are a proprietary activated polyamide which reacts with amine functions on the protein. These membranes do not appear to exhibit significant permselectivity for small molecules.

A widely employed pre-cast material is cellulose acetate available as dialysis membrane and in the form of hollow fibres (Amicon). The polymer possesses some negative charge derived from the presence of residual carboxyl groups. At physiological pH these are ionized. Consequently CA membranes not only exclude proteins but are capable of retarding the transport of anionic species such as ascorbate and urate, two major electrochemical interferents, particularly when hydrogen peroxide is monitored. The actual selectivity depends on membrane thickness and preparation procedure, but *Table 1* gives some data that shows the magnitude of interferences for a glucose oxidase-based sensor particularly as applied to blood serum measurements. It is possible to cast membranes as thin as 5 μm using *Protocol 5*. If a spreader is not available, it is possible to cast a film on a glass plate by drawing a circle on the plate with a wax pencil. Depending upon the area chosen, a known volume of the CA solution is pipetted on the plate so as to produce a film of reproducible thickness. After the solvent evaporates, the film can be removed as described

Table 1. Substances interfering with glucose sensor response.

Substance	Interfering level[a] (mg dl^{-1})	Serum level (mg dl^{-1})
Acetone	26000	0.3 – 2.0
Beta hydroxybutyric acid	14000	–
Sorbitol	14000	–
D-xylose	730	–
D(–) adrenaline	110	–
Ascorbic acid	280	0.4 – 1.5
L(+) cysteine.HCl	100	0.9
D(–) fructose	5400	<7.5
d-Galactose	300	<20.0
Glutathione	100	28 – 34
d-Mannose	170	–
Tyrosine	160	0.8 – 1.3
Uric acid	400	3 – 7
Acetaminophen	1.5	–
Acetylsalicylic acid	167	–
Catechol	0.3	–
Sodium oxalate	11000	–
Heparin sodium	1800 U ml^{-1}	–
Sodium azide	360	–
Thymol	75	–
Epinephrine	–	18 – 26 ng dl^{-1}
Norepinephrine	–	47 – 69 ng dl^{-1}

[a] Corresponds to the level of interferent which would give an error of 5 mg dl^{-1} in an apparent glucose response. Measured with a Yellow Springs Instruments Model 2300 sensor. Interference data courtesy of YSI, Inc.

in the protocol. It should be pointed out that the membrane pore size is very dependent upon the solvent composition including water content, the rate of evaporation, the humidity and the temperature of the deposition environment (12). To obtain a reproducible product, it is therefore necessary to control these parameters carefully.

Three polymeric materials lend themselves well to deposition directly from solution: cellulose acetate, Nafion and polyurethane. The former material can be deposited on a sensor surface by dip coating using the solution described in *Protocol 5*. Nafion, a perfluorosulphonic acid ionomer made by DuPont is available in a low equivalent weight form (eq. wt 1000) which is soluble in low molecular weight alcohols. It can also be obtained as a 5% (w/w) solution in alcohol from Aldrich. By virtue of the negative charge created by the presence of sulphonate groups, a membrane fabricated from this material is capable of concentrating cationic species and excluding anions. These membranes have been deposited on surfaces in thicknesses as small as 1000 Å (13) and have been studied extensively in a variety of electrochemical applications (14,15). Nafion films can be deposited by dip coating with the 5% ionomer solution. Nafion films tend to adsorb proteins and other cationic species readily, and these may interfere with sensor response. Consequently, they are most effectively employed when coated with an external polymer layer such as polyurethane or CA which is more inert in this respect.

There have been numerous reports in the literature (16) involving the use of

polyurethane (PU) as a biocompatible material. These have included its use as a protective membrane on implantable glucose sensors (17–18). Unfortunately, commercially available PU is produced in widely varying weight-average molecular weights which possess different functional groups. Thus the transport properties of this material as a film will differ considerably from source to source. Linear segmented aliphatic polyether-based polyurethane (EG80A or SG85A) is available from Thermedics Inc. A protective coating can be applied to a sensor by the following procedure.

Protocol 7. Application of a polyurethane protective coating

1. Prepare a mixture of 98% tetrahydrofuran and 2% dimethylformamide (v/v) and dissolve polyurethane in it to a final concentration of 4% (w/v).
2. Dip the tip of the sensor in the polyurethane solution and then remove it and allow the solvent to evaporate at room temperature.
3. Store the sensor in 0.1 M phosphate buffer, pH 7.4 for 2 days at room temperature prior to use.

PU is useful as an outer protective coating. If the PU solution is applied to a sensor surface which already has a cellulose acetate film and/or enzyme layer on it, care must be taken to ensure that the base films are not disrupted by the PU application. This is best accomplished by making one quick dip of the sensor into the PU solution. PU exhibits some permselectivity to small molecules and retards glucose access to the enzyme layer. This lowers sensitivity but leads to a sensor with a wide linear dynamic range (18).

2.3 Cell and sensor geometry

As most enzymatic reactions used for enzyme electrodes are irreversible, these biosensors deplete the substrate at their surface. Thus the supply of substrate to the sensor surface will be affected by hydrodynamic conditions in its vicinity. It is important to control solution flow by stirring (probe sensor) or by circulation of the sample solution (flow through sensor). Alternatively, the sensor may be rotated in the test solution. In all cases, the enzymatic membrane or layer, possibly also covered by a protective membrane, must be maintained in close proximity with the platinum working electrode. This positioning can be maintained with a screw cap (Radiometer Tacussel Type GLUC-1) or by the spacer of a modified liquid chromatography electrochemical detector (Radiometer Tacussel Type DEL-1) shown in *Figure 3* (19). In the latter case, solution is circulated through the cell using a Gilson Minipuls II peristaltic pump at a flow rate ranging from 0.1 to 2 ml min^{-1}. Auxiliary and reference electrodes are included in the sensor for control of applied potential. They are generally situated on the same side of the membrane as the working electrode in order to avoid resistive potential drop across the membrane.

Microsensors may be fabricated in a needle configuration (18).

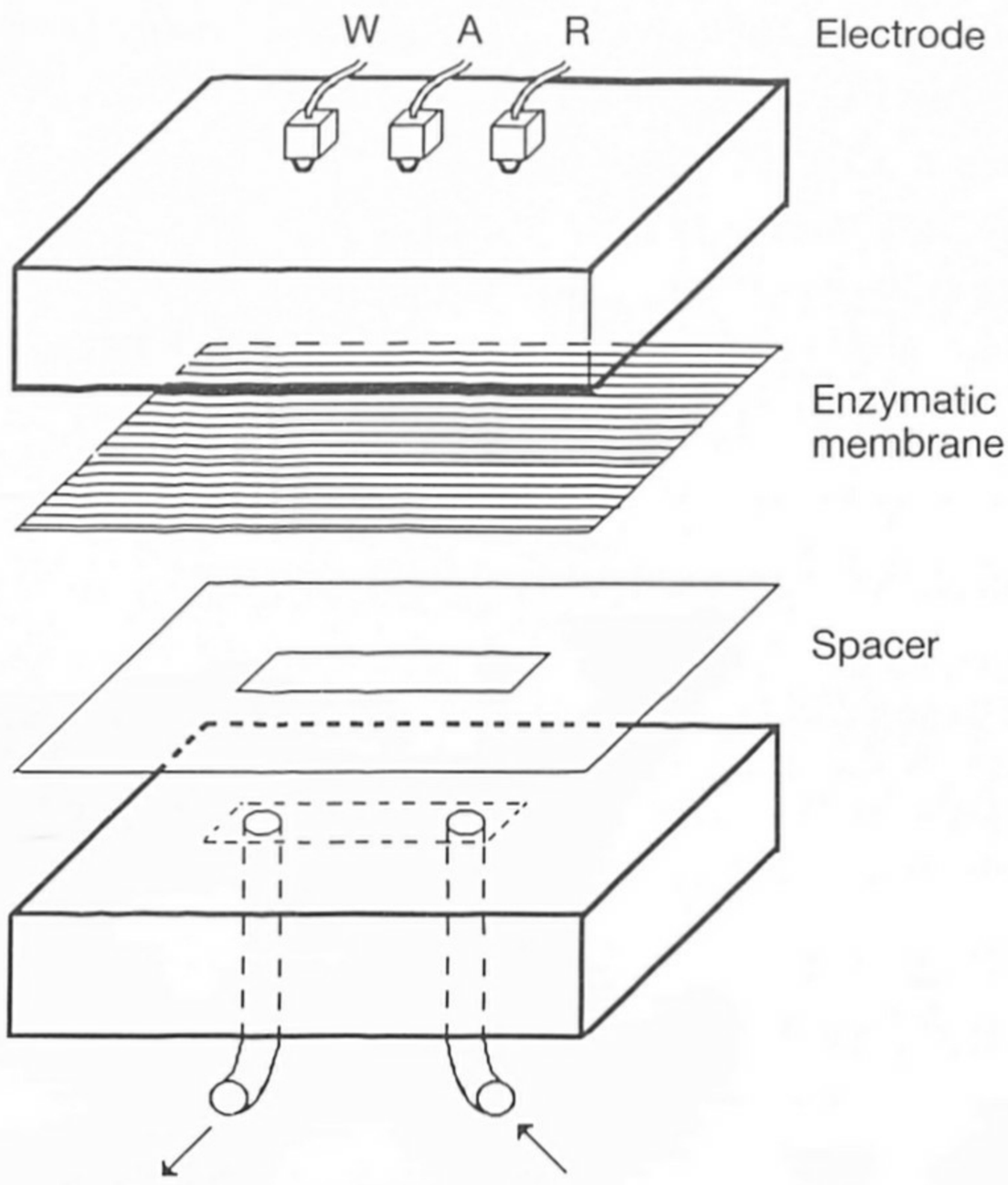

Figure 3. Schematic diagram of flow-through enzyme electrode. W, working electrode; A, auxiliary electrode; R, reference electrode.

Protocol 8. Fabrication of a needle-type microsensor

1. Heat the tip of a platinum wire (200 μm diameter, 10 cm length) to about 2450°C with an oxygen–butane microtorch to form a small sphere with a surface area of 1–2 mm^2.
2. Seal the platinum wire into a polyethylene catheter (i.d. 0.3 mm, Biotrol Pharma) with epoxy cement.
3. Take a 23 gauge hypodermic needle (1.24 mm i.d.) and cut the end off square. Thread the catheter through the needle so that the platinum ball is held against its end and cover the inner half of the ball with epoxy cement.
4. Place the needle in a support and rotate it at 13 r.p.m. for 48 h to allow the epoxy to harden.
5. Wash the tip of the platinum sphere with trichloroethylene and then dip it in an ultrasonic bath of distilled water for 5 min to remove organic deposits.

Note: the area of the sphere can be conveniently determined with a micrometer.

The deposition of the enzyme and polymeric layers can then be carried out according to the procedures outlined in Sections 2.1 and 2.2.

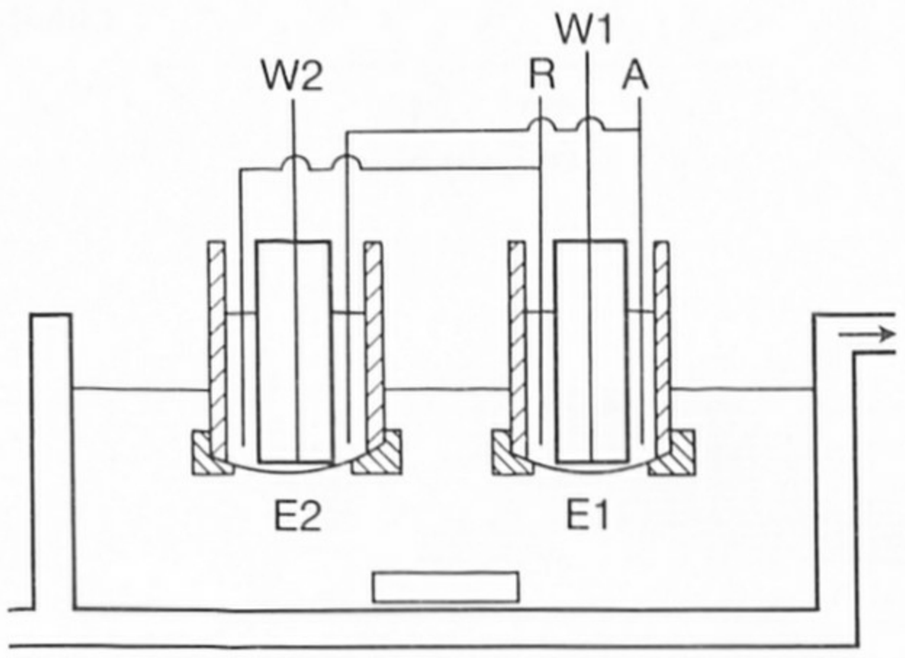

Figure 4. Schematic diagram of differential sensor apparatus. E1, enzymatic probe; E2, non-enzymatic (compensating) probe; W1,W2, working electrodes; A, auxiliary electrode; R, reference electrode.

2.4 Instrumentation

2.4.1 Principles

For the operation of a probe amperometric enzyme electrode, the following equipment is necessary: a thermostatted cell with magnetic stirrer, a sensor body, a potentiostat with amperometric readout, and a chart recorder. The temperature of the solution and of the sensor itself should be carefully controlled. The sensor sensitivity can vary by 3−10% per °C (20). Temperature also affects oxygen solubility and therefore the rate of enzymatic reactions that depend on oxygen such as those catalysed by oxidases. Control to ±0.1°C with a thermostatted cell and water bath is usually sufficient. It is important that all solutions i.e. buffer, standard and sample be brought to thermal equilibrium prior to measurement.

Protective outer membranes may not exhibit sufficient permselectivity to exclude all interfering electroactive species especially those which are uncharged. It is therefore necessary to make a differential measurement using dual sensors: an enzymatic and a non-enzymatic (compensating) element. Such a configuration has been found very useful for glucose determinations in food and clinical samples (8). The differential mode is illustrated in *Figure 4*. Sensors E1 and E2, corresponding to the enzymatic and compensating elements, respectively, are dipped into the same test solution. A potentiostat is used to maintain a potential difference of 650 mV between working electrodes W1 and W2 and the reference electrode, R, typically Ag/AgCl. The current outputs from W1 and W2 are first subtracted and then differentiated. Four time-dependent signals are thus available: $I1$, $I2$, $(I1-I2)$, and $\mathrm{d}(I1-I2)/\mathrm{d}t$ versus time. If a four electrode system such as that used for ring-disk voltammetry (Radiometer-Tacussel, BIPAD) or a dual electrode elelctrochemical detector for liquid chromatography (Bioanalytical Systems, Inc., LC-4B) or differential current amplifier (Radiometer-Tacussel, DELTAPOL) is unavailable, then it will be necessary to use two single potentiostats. There is a tendency for

the two systems to interact electrically when common auxiliary and reference electrodes are used, so it is necessary to verify that the W1 and W2 outputs vary independently by addition of glucose and hydrogen peroxide or ascorbate, respectively. If only one potentiostat and working electrode is available, then a background current, *Ib* is determined in the absence of substrate. Subsequently analogous determinations of $I1$, $(I1-Ib)$ and $\mathrm{d}(I1-Ib)/\mathrm{d}t$ can be made.

Indicating (working) electrodes are generally made in the form of a platinum disk, wire, or foil. This material has been found to be better than gold or carbon for hydrogen peroxide detection. When oxygen is monitored, platinum or gold electrodes may be used alternatively.

The most widely used reference electrode is silver/silver chloride (Ag/AgCl) which can be prepared as a disk, ring or wire. Chloridation of the silver surface is easily performed by anodic oxidation under constant current or potential. Constant current is preferred because a more uniform and reproducible electrode usually results. The oxidation is carried out in 0.1 N HCl for 30 min at a current density of 0.4 mA cm^{-2} (21). Further details on the preparation of this type of electrode can be found in *Protocol 7* of Chapter 3.

When small indicating electrodes are used and consequently currents below 0.1 μA are measured, the usual three-electrode potentiostat (working, auxiliary and reference electrode) may be replaced by the simpler two-electrode (working and reference) system. In the latter case the reference electrode acts also as the auxiliary electrode and must maintain a stable potential even when current is passing through it. Possible potential variation in the reference electrode may be minimized by making its area ideally 4–5 times larger than that of the indicating electrode.

2.4.2 Assembly of low cost systems

The sensor body may be easily prepared by modifiction of a conventional gas electrochemical sensor. The hydrophobic membrane of a Clark-type oxygen sensor can be replaced or covered with enzymatic and protective membranes. An ammonia or carbon dioxide sensor can be used if the pH detector is replaced by a platinum disk working electrode covered with an enzymatic membrane (Model 8002-2 ammonia electrode—ABB Kent). Sensor bodies specifically designed for enzyme electrodes may be obtained from the following firms: GLUC-1 sensor (Radiometer-Tacussel), three-electrode probe Model 110708 (available in limited quantities—Yellow Springs Instruments, Inc.).

Although it is easy to design and build a single or differential potentiostat and amperometric unit using operational amplifiers, one may alternatively purchase these items. Potentiostats designed for liquid chromatographic detectors are quite suitable for this purpose becaues they can measure the small currents (microamperes to nanoamperes) characteristic of microsensors. Typical items are available from: Radiometer-Tacussel (Model PRG-DEL or PRG-GLUC) and Bioanalytical Systems, Inc. (Model LC4B). General-purpose or specifically designed workstations (22) can facilitate data acquisition and processing especially when numerous measurements are made.

2.4.3 Commercially available systems

There are integrated systems available which incorporate the sensor, readout device, temperature control, stirring and data acquisition into the same unit. These instruments are designed around specific analytes, but the enzymatic membrane provided can be replaced with one prepared using the methods described in Sections 2.1.1 and 2.1.3 allowing alternative analytes to be measured. Some sources of commercial instruments are: Yellow Springs Instruments, Inc. (Model 2000—detects hydrogen peroxide); Radiometer-Tacussel (GLUCOPROCESSEUR—detects hydrogen peroxide with differential electrodes); SERES (ENZYMAT—detects oxygen); SGI (MICROZYM-L—detects ferrocyanide).

2.5 Characterization of sensor response

2.5.1 Evaluation of sensitivity, stability, linearity and response

Calibration of the sensor is made by adding standard solutions of the analyte and is carried out in either of two modes depending upon whether the steady-state or dynamic response is measured as described below.

Protocol 9. Sensor calibration procedure

1. Dip the sensor into a thermostatted cell (at 37°C) containing 25 ml of buffer at the pH and ionic strength for optimal enzyme activity.
2. Apply the appropriate potential to detect the species of interest (+650 mV versus a Ag/AgCl reference electrode for hydrogen peroxide) and wait for the background current to stabilize. This takes typically about 20 min.
3. Add aliquots (25−125 μl) of standard analyte solutions (concentrations of 0.01−1 M) to generate a series of concentration steps.
4. Measure either the plateau current attained (steady-state response) or the maximum rate of change of the current from the derivative of the current−time curve (dynamic response).

The steady-state response is defined by the plateau reached in monitoring ($I1-Ib$) or ($I1-I2$) as a function of time. The dynamic response is obtained as the maximum of the current derivative i.e. $\mathrm{d}(I1-I2)/\mathrm{d}t_{max}$ or $\mathrm{d}(I1-Ib)/\mathrm{d}t_{max}$. The latter response can be measured more rapidly and thus improves overall sample measurement throughput. Dynamic response is proportional to the increase in substrate concentration in the reaction vessel (8) and this principle is frequently exploited in automated systems.

Steady-state responses are calculated by comparing the steady-state current either to the background current (Ib) in the absence of substrate or to the steady-state current corresponding to the previous addition. Thus either $I-Ib$ versus concentration (C) or delta.I/delta.C versus C or log C curves are plotted. Sensor sensitivity is best evaluated by measurement of delta.I/delta.C for each value of C in the cell. It is

generally possible to measure the steady-state and dynamic responses over a large range of analyte concentration and successive substrate determinations are possible every 1−3 min by washing the sensor or rinsing the cell. If washing is required, it will be necessary to wait several minutes for the current response to return to the background levels. To facilitate comparison of sensors with different geometries, the observed sensitivity should be divided by the working electrode area (A), i.e. $(I-Ib)/A$ or (delta.I/delta.C)/A.

The limit of detection can be determined by comparison of background signal fluctuations and signal response. A signal/noise ratio of 2 is usually chosen as the limit definition. For very dilute solutions, i.e. 10−100 nM, the precision for the determination of substrate depends on the noise level, which is somewhat less for the steady-state than for the dynamic response. Probe electrodes generate less noise than flow through sensors because of the pulsation in flow rate created by the peristaltic pump in the latter case.

The linear range of the calibration curve is determined by plotting delta.I/delta.C versus C or by comparing delta.I/delta.C values for successive substrate additions. This method is much more definitive than plotting the usual calibration curves, $I-Ib$ versus C. The linear range usually extends over two orders of magnitude, between approximately 10 μM and 1 mM. When large working electrode areas are used, it is possible to obtain sensors linear between 100 nM and 3 mM (8). Response times are determined for each substrate pulse into the cell and are measured to 90 or 95% of the steady-state response. For the dynamic response the maximum value of the first derivative is used to define the response time. It is important to ensure that solution mixing or the time constant of the measurement electronics does not define the overall response.

Stability of sensor response may vary considerably depending on the sensor geometry, preparation method, and enzyme used. Sensors have been reported usable for periods of more than one year (23). How the sensor is stored and how frequently it is used will have important influences on its useful lifetime.

2.5.2 Assessment of specificity and interferences

Selectivity depends first upon the enzyme chosen. Most enzymes, except alcohol or amino acid oxidases, are very specific. Thus sensor E1 (*Figure 4*) yields a high selectivity for substrate. For example, glucose oxidase is 5×10^4 times more active with glucose than with other sugars such as fructose, lactose, or sucrose.

The main interference, therefore, is derived from electroactive species which can diffuse to the sensor surface to be oxidized. This is particularly a problem when the relatively high potential (+0.65 V versus Ag/AgCl) required to oxidize hydrogen peroxide is applied. This has led some investigators to suggest using a much lower potential (−0.4 V versus /Ag/AgCl) to monitor the oxygen decrease as a measure of substrate concentration. This can be done, but determination of the background signal is much more difficult. By use of the compensating electrode, E2, interference from such species as ascorbate, urate and tyrosine can be eliminated. For example, the selectivity coefficient for glucose-dependent hydrogen peroxide over non-

enzymatically generated hydrogen peroxide is between 4×10^{-3} and 1.3×10^{-2} depending upon experimental parameters (8).

Assessment of selectivity is determined by comparing sensitivities (within the linear range of the calibration curve) for substrate and interferents. The parameters are calculated as (delta.I/delta.C_{subst})/(delta.I/delta.C_{interf}).

3. Conclusions

There are a large number of possibilities for the application of unmediated amperometric enzyme electrodes. A perusal of the literature will indicate that the vast majority of applications have involved either glucose or lactate as substrates. This is partly because the enzyme electrode is probably the method of choice in these cases and because these analytes are of considerable biomedial interest. One can envisage a variety of other applications particularly where the analyte is in the concentration range of millimolar to micromolar, where the sample matrix is complicated and where it is not desirable or possible to make a separation prior to analysis.

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